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Epigenetics in eating disorders: a systematic review

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Abstract

Eating disorders are complex heritable traits influenced by both genetic and environmental factors. Given the progress of genomic discovery in anorexia nervosa, with the identification of the first genome-wide significant locus, as well as animated discussion of epigenetic mechanisms in linking environmental factors with disease onset, our goal was to conduct a systematic review of the current body of evidence on epigenetic factors in eating disorders to inform future directions in this area. Following PRISMA guidelines, two independent authors conducted a search within PubMed and Web of Science and identified 18 journal articles and conference abstracts addressing anorexia nervosa ($n = 13$), bulimia nervosa ($n = 6$), and binge-eating disorder ($n = 1$), published between January 2003 and October 2017. We reviewed all articles and included a critical discussion of field-specific methodological considerations. The majority of epigenetic analyses of eating disorders investigated methylation at candidate genes ($n = 12$), studying anorexia and bulimia nervosa in very small samples with considerable sample overlap across published studies. Three studies used microarray-based technologies to examine DNA methylation across the genome of anorexia nervosa and binge-eating disorder patients. Overall, results were inconclusive and were primarily exploratory in nature. The field of epigenetics in eating disorders remains in its infancy. We encourage the scientific community to apply methodologically sound approaches using genome-wide designs including epigenome-wide association studies (EWAS) as well as broaden the focus to include studies of all eating disorder types.

1 INTRODUCTION

Eating disorders are serious illnesses associated with significantly reduced health-related quality of life [1,2]. Our current understanding of their etiology is piecemeal and the evidence base for their treatment is inadequate [3]. Over the past two decades, family, twin, and adoption studies have robustly shown that eating disorders are complex traits influenced by both genetic and environmental factors. Twin-based heritabilities for anorexia nervosa (AN) range from 48% to 74%, for bulimia nervosa (BN) from 55% to 62%, and for binge-eating disorder (BED) from 39% to 45% [4]. A genome-wide association study of AN has yielded the first genome-wide significant locus on chromosome 12—a chromosomal region previously associated with autoimmune diseases including type 1 diabetes [5]. AN, furthermore, shows significant genetic correlations with various psychiatric, personality, and metabolic phenotypes, including schizophrenia, neuroticism, glucose, and lipid metabolism. This panel of findings has encouraged a reconceptualization of AN as both a metabolic and psychiatric disorder [6]. We anticipate an accelerated discovery trajectory of common genetic variation in AN and extensions to BN and BED are underway by members of the Eating Disorders Working Group of the Psychiatric Genomics Consortium (<http://pgc.unc.edu>). This foundational work sets the stage for a systematic review of another branch of genetic research, namely, epigenetics.

The genetic variants contributing to the majority of disease liability in eating disorders have yet to be identified, and non-genetic factors should continue to be explored—indeed monozygotic twins who are discordant for eating disorders have been reported, suggesting that genes do not act alone in eating disorders risk [7,8]. Other important and poorly understood features are the uneven sex distribution of eating disorders [9], and the peak age of onset (especially in AN and BN) hovering around adolescence and puberty [10], a period characterized by considerable

hormonal transformation, leading to widespread epigenetic and gene expression changes [11], and considerable social and emotional change [12,13]. The extreme dietary insults experienced by individuals with eating disorders (i.e., prolonged restriction, or intermittent binge eating and fasting) also have the potential to cause both temporary and longer lasting epigenetic changes, which may be important in treatment response and relapse.

Complementing genetic studies of eating disorders, epigenetic mechanisms have spurred much interest in recent years, offering an added layer of gene regulatory information, which could link external and internal environmental stimuli as well as non-coding genetic variation with transcriptional consequences, altering downstream phenotypes. Together with an increasing understanding of the genetic variants underlying heritable disease risk in eating disorders, epigenetics has the potential to aid in disentangling the molecular genetic pathways that contribute to the development and progression of the illnesses.

In the context of this review, epigenetics refers to various biochemical mechanisms giving rise to changes in gene regulation, which are either heritable or characterized by long-term stability [14]. Effectively, this means that epigenetic mechanisms regulate gene expression, and that the regulatory features could be copied and transmitted at cell division or could exhibit relative stability in the cellular environment [15]. For example, pluripotent stem cells can develop into a variety of different cell-types which are characterized by vastly different gene-expression profiles. But as cells progress further along the differentiation cascade (a process largely driven by epigenetic mechanisms), their gene-expression and epigenetic profiles become ever more stable or “locked in” [16]. While historically defined as occurring independent of the DNA sequence, recent work has provided evidence for widespread effects of genetic variants on epigenetic states. In particular, methylation quantitative trait loci (mQTLs) are increasingly being

characterized: single nucleotide polymorphisms (SNPs) that exert influence on the methylation state of a CpG site, usually in close vicinity to the SNP [17,18]. At the same time, epigenetic marks have been shown to be responsive to several environmental stimuli. Perhaps most strikingly this has been shown for tobacco smoking, which was found to have considerable effects on DNA methylation across several genomic regions [19]. Similarly, epigenetic profiles are highly correlated with chronological ageing and a strikingly accurate predictor of age has been derived based on the DNA methylation profiles of only around 300 CpG sites [20].

Biologically, epigenetic mechanisms can be categorized into three groups: DNA modifications, histone modifications, and non-coding RNA (Figure 1).

DNA modifications are chemical modifications that bind to the DNA itself. Most prominently and prevalently, this occurs in the form of DNA methylation, the addition of a methyl-group to cytosine, in the context of cytosine-guanine dinucleotides (CpG sites). DNA methylation is the best studied epigenetic mechanism in the context of complex diseases thus far and disease-associated methylomic dysregulation has been reported for a number of psychiatric disorders, including schizophrenia [21,22], Alzheimer's disease [23,24] and autism spectrum disorder [25,26]. In addition to DNA methylation, its oxidized derivatives constitute further DNA modifications, with DNA hydroxymethylation generating increasing interest in the context of neuropsychiatric disease, due to its enrichment in the human brain [27,28].

Histone proteins constitute the cores around which DNA is wrapped in the cell nucleus. They can exert an effect on gene regulation by altering the accessibility of DNA sequences, primarily via chemical modifications to the N-terminal histone tails, which extend out of the nucleosome complex. An increasing number of modifications to amino acids in the histone tails are being identified, including methylation, acetylation, and phosphorylation. These modifications are

characterized by tissue-specificity and are highly correlated with different transcriptional chromatin states [14].

Finally, non-coding RNAs—expressed transcripts which do not code for proteins—have widespread effects on gene regulation via mechanisms including post-transcriptional silencing, for example by binding to transcripts and inhibiting their translation into proteins [29,30], or chromatin remodeling, for example by affecting the positioning of nucleosomes along the genome and thereby altering the accessibility of specific DNA sequences [31].

1.1 Epigenetic research in eating disorders

Eating disorders exhibit several characteristics not in line with Mendelian inheritance, including sex differences (i.e., females are 8 times more likely to suffer from AN or BN than males) [9] and periods of increased risk of onset (i.e., particularly in adolescence and young adulthood) [10], as well as reported discordance between monozygotic twins [7,8]. These epidemiological characteristics make them an excellent target for the examination of epigenetic effects on appetite regulation and eating behavior. Epigenetic research in eating disorders has so far focused exclusively on DNA methylation, using three different approaches to investigate disease-associated methylomic variation. First, early DNA methylation studies measured global methylation levels in eating disorder cases comparing them with methylation levels in healthy controls. Second, DNA methylation at selected candidate genes has been assessed. These candidate gene studies rely on previously established links between a gene and the phenotype, for example from proteomic investigations or previous insights into biological pathways involved in a phenotype. The third approach encompasses all methods allowing for genome-wide coverage of DNA methylation profiles and does not rely on any prior hypotheses about the

nature of possible associations. In general, hypothesis-free approaches that explore the whole genome are the gold standard in genetic research. Genome-wide approaches are applied in the investigation of common genetic variation (i.e., single nucleotide polymorphisms, SNPs) in genome-wide association studies (GWAS) as well as in the examination of epigenetic alterations in epigenome-wide association studies (EWAS). EWAS refers to the site- or region-specific investigation of epigenetic profiles at a genome-wide level. While technically only whole-genome next generation sequencing-based approaches cover the majority of the human genome, array-based technologies are also labelled as genome-wide, due to coverage of loci mapping to most genes and genomic regions across the genome. Most often, EWAS investigate phenotype-associated variation in DNA methylation in a site-specific manner using microarrays, laboratory tools that allow researchers to study the methylation profile of thousands of CpG sites across the human genome simultaneously. Statistically, EWAS share similarities with GWAS in that site-specific associations with a phenotype across a large number of genomic loci are conducted. However, unlike the genome sequence, epigenetic marks are dynamic and can vary across cell- and tissue-types, age and development, and are subject to environmental confounders including smoking, medication, and stress (Figure 2).

In this sense they are more accurately characterized as intermediate biological phenotypes and are susceptible to confounding and other problems faced in traditional observational studies. This phenotypic nature of epigenetic profiles means that sources of variation or confounding need to be taken into account in the experimental design and statistical analyses [32,33]. For example, if all individuals in the control group are older than the affected individuals, an EWAS may detect epigenetic differences relating to ageing between the two groups, rather than differences associated with disease status.

2 METHOD

2.1 Search strategy

Our systematic literature review was conducted according to the PRISMA guidelines [34]. We conducted an exhaustive literature search using the electronic databases PubMed and Web of Science with a time limitation starting with articles published after 01.01.2003. We used following key search terms including (anorexia OR bulimia OR “binge-eating disorder” OR “eating disorder”) AND (epigenetics OR methylation OR histone OR “non-coding RNA”). The search was repeated by the co-primary author to avoid selection bias. Furthermore, we screened the references of published articles and reviews. Our search results including the selection process are presented in Figure 3 according to PRISMA guidelines.

2.2 Selection criteria

Our inclusion criteria were as follows:

- a. Studies investigating humans only
- b. Any age group
- c. Clinical diagnoses of AN, BN, or BED
- d. Investigation of any type of epigenetic mechanism: methylation, histone modification, non-coding RNAs
- e. Published after 01.01.2003
- f. Study includes a control group or comparison group
- g. Publications in any language

2.3 Data extraction

We extracted following information from every identified study:

- a. Author
- b. Publication year
- c. Sample
- d. Follow-up period
- e. Diagnostic criteria
- f. Participant screening and exclusion criteria
- g. Number of cases (AN, BN, BED)
- h. Number of controls
- i. Matching of cases and controls
- j. Outcome variables (Genome-wide methylation level, candidate genes, number of CpG sites)
- k. Covariates
- l. Tissue
- m. Correction for multiple comparison
- n. Laboratory methods
- o. Limitations

3 RESULTS

A total of 178 papers were identified by our search terms. We excluded 67 studies because they did not cover eating disorders, six did not investigate humans, 19 were reviews, one was a book chapter, and three did not examine epigenetic mechanisms. This resulted in 16 published studies and two conference abstracts on epigenetics that met criteria for inclusion in our systematic review (Figure 3). One full-text article was a duplicate of a conference abstract, resulting in 17 studies. To our knowledge these represent all published studies and conference abstracts investigating epigenetics in eating disorders that were available at the close of our search in October, 2017. We contacted authors of conference abstracts for additional information on their studies (Table 1).

[Insert Table here]

3.1 Recent body of evidence

To date, 17 studies on the epigenetics of eating disorders have been published of which four investigated global DNA methylation levels, 12 investigated candidate genes, and three used microarray-based technologies to profile DNA methylation across the human genome. All studies but one were cross-sectional. All studies focused exclusively on DNA methylation and some also investigated expression levels, but did not investigate other epigenetic mechanisms, such as histone modification or non-coding RNAs. The studies show extensive sample overlap as four studies are part of the homocysteine and DNA methylation in eating disorders (HEaD) study [35–38], two studies recruited inpatients at the Universitätsmedizin Charité Berlin, Germany [39,40], and four studies recruited at the Douglas Institute Eating Disorders Program in Montreal, Canada, [41–44].

3.2 Global DNA methylation levels

Four studies investigated global DNA methylation differences in eating disorders. All studies focused on AN [38,44–46], with one study also investigating BN [38]. Two studies reported global hypomethylation in individuals with AN [38,46], one study reported global hypermethylation in AN [44], and one reported no difference in global DNA methylation levels between AN cases and controls [45]. Patients suffering from BN showed no difference in their global DNA methylation levels compared with controls [38].

Limitations of global DNA methylation studies. Overall, these study results were inconclusive and did not reveal a clear and replicable global DNA methylation pattern in either AN or BN. All four studies were small, with the largest study profiling 32 AN cases and 24 BN cases, respectively, substantially limiting the power to detect effects. More generally, global levels of DNA methylation may not be of much relevance to epigenetic epidemiology, as they fail to provide information on region-specific DNA methylation, and lack the specificity to associate the dysregulation of biological pathways with the occurrence of a disease [47]. Even within the framework of global DNA methylation studies, the methods employed in these four studies limit the examination of DNA methylation to either promoter regions (for the approaches based on methylation sensitive restriction enzymes) or LINE1 elements [45], overlooking other parts of the genome.

3.3 Candidate gene studies

Candidate gene studies are hypothesis-driven and investigate DNA methylation in the vicinity of selected genes. These candidate genes are selected based on prior knowledge, for example, following differences in protein levels measured in clinical studies assessing patients with AN or

BN. Overall, 12 studies have been published profiling DNA methylation in candidate gene regions in the context of eating disorders, 11 of which focus on AN and BN. These eleven studies investigated genes relating to synaptic transmission [38], endoplasmatic reticulum stress response [38], fluid balance [35], the cannabinoid system [36], dopamine transmission [37,43,48], stress response [39,41], appetite regulation [39,42,48], serotonin transmission [48], and oxytocin [49]. One methylomic study of candidate genes in BED has been reported [50,51]. The study primarily investigated promoter methylation of *SLC1A2*, a gene involved in glutamate clearance, in bipolar disorder (BD). The authors found decreased DNA methylation in BD patients who also suffer from BED, compared to those that are only affected by BP. However, their sample of patients suffering from binge-eating behavior seems to comprise BN and BED cases, rendering the interpretation of the results ambiguous [50,51]. All candidate gene studies of eating disorders are described in detail in Table 1.

Limitations of candidate gene studies. Across these studies, no clear differentially methylated candidate genes for AN, for BN, or for BED were robustly identified. Most candidate regions were only profiled once, and results of repeatedly measured genes did not replicate across the different studies, showing no clear eating disorder-associated methylomic variation across the selected candidate genes. In addition to non-replication, these studies were limited by small sample sizes: most of the study populations included on average only 30 cases with one study including 64 cases [42] and another 206 cases [43]. Furthermore subjects occasionally comprised a mixture of acutely ill and recovered patients [49] or a mixture of different eating disorders [50,51]. This is particularly concerning as dietary changes, weight changes, and accompanied alterations of hormonal levels during the recovery process can have a major effect on epigenetic profiles in individuals with eating disorders.

In epigenetics, as in genetics, a general drawback of a candidate gene approach is their hypothesis-driven design. Specific genes are selected for investigation based on prior knowledge, narrowing the investigation to only a very limited part of a large system and ignoring the majority of other genomic regions. Methodological shortcomings, such as the failure to correct for multiple comparisons, or failing to account for factors that influence DNA methylation, for instance diet and smoking, pose additional limitations to candidate gene studies. Further possible confounders are discussed below.

3.4 Epigenome-wide association studies (EWAS)

Three EWAS investigated genome-wide DNA methylation profiles in AN using the Illumina Infinium® HumanMethylation450 BeadChip. No genome-wide studies of DNA methylation have been published on other eating disorders. Booij et al. [44] reported 14 differentially methylated CpG sites comparing 29 AN patients with 15 normal-weight controls. These 14 hypermethylated CpG sites were annotated to 11 genes (*PRDM16*, *HDAC4*, *TNXB*, *FTSJD2*, *PXDNL*, *DLGAP2*, *FAM83A*, *NR1H3*, *DDX10*, *ARHGAP1*, *PIWILI*) [44]. Kesselmeier et. al [7] reported 51 differentially methylated CpG sites when comparing 22 AN cases with 24 lean individuals and 81 CpG sites when comparing AN cases with 30 individuals from a general population sample. They also showed that 54 of the 81 sites exhibited directionally consistent differential DNA methylation differences in a comparison of twins discordant for AN assessed by a binomial sign test. While the authors report a replication of hypermethylation previously reported at a CpG site annotated to *TNXB* [7,44], the significance level for this replication was only suggestive. In this study, controls recruited from the population were on average older than the AN patients which can confound the results as methylation patterns are age-dependent [7,20].

In a conference presentation, Ramoz et al. [52] conducted the only longitudinal investigation of 36 acutely ill AN patients of whom half remitted after one year. No significant differences in DNA methylation emerged between remitted AN patients and those patients who were still ill after a follow-up period of one year. However, the study does not include a control group [52].

Limitations. While EWAS report significant findings of identified differentially methylated CpG sites comparing patients suffering from AN with controls, they are limited by several experimental and methodological factors. First, the reported samples never included more than 29 cases of AN which is far too small to robustly detect patterns of differential methylation at a genome-wide scale, i.e. when conducting over 450,000 statistical tests [53]. Secondly, multiple testing correction was not always performed stringently, e.g., when “suggestive” significant results are reported or examined sites are filtered before or after analysis based on methylation variability. Thirdly, when studying associations between eating disorders and DNA methylation, potential confounding factors, including age, weight, diet, and medication need to be accounted for, as outlined in more detail in the discussion. As such, many of the EWAS included above labeled as pilot studies by the authors, provide motivation for further investigation, and are a springboard to launch full-scale projects with larger sample sizes and careful study setup, data collection, and analysis. Future studies will also require replication in independent samples and should adhere to stringent methodological criteria, including multiple test correction, no subjective filtering of CpG sites, and controlling for confounding factors.

4 DISCUSSION

Short summary. The current research on epigenetics in eating disorders is limited and not yet sufficiently mature to draw sound conclusions. Studies investigating global DNA methylation levels and candidate gene studies are inconclusive and suffer from non-replication, in addition most candidate genes have only been examined once. The DNA methylation levels of candidate genes involved in dopamine signaling, which were measured repeatedly, did not replicate [37,43,48]. EWAS examining CpG sites at a genome-wide level have identified multiple AN-associated differentially methylated sites, replicating a differentially methylated position at *TNXB* in one independent study [44]. However, the hypermethylation at this CpG site annotated to *TNXB* only reached suggestive significance in the replication attempt [7], failing to survive stringent correction for multiple comparisons. A false positive finding, therefore, cannot be ruled out. Nonetheless, these suggestive findings from the four published epigenome-wide studies, notwithstanding their small sample sizes, encourage further work. They provide motivation for rigorous investigation of epigenetic mechanisms in the etiology of eating disorders in much larger samples, because the lack of convincing evidence is most likely linked to small sample sizes drastically limiting power to detect differences in genome-wide epigenetic profiles. It will, however, be crucial to carefully design future epigenetic studies of eating disorders and adhere to stringent methodological standards to generate widely accepted and robust results.

4.1 Future directions

Sample size. One of the primary goals of future epigenetic investigations of eating disorders should be to increase sample sizes to improve the power to detect effects, even when effect sizes are small. Recent epigenetic studies of other psychiatric disorders and environmental exposures

have examined epigenetic differences in samples comprising thousands of participants and notably, replicated successes have been documented for a number of exposures and diseases including tobacco smoking [19], C-reactive protein levels in serum blood [54], and Alzheimer's disease [23,24]. It is important to foster international collaborations in epigenetics to collectively achieve this aim. While several large consortium efforts have led to advances in characterizing baseline human tissue epigenomes [14,55,56], this is rarely extended to the realm of epigenetic epidemiology in complex diseases. Analogous to the collaborative success of the Psychiatric Genomics Consortium in genetic research, a concentrated international research effort could facilitate discovery in epigenetic epidemiology [57]. Unique challenges do exist in conducting large-scale collaborative epigenetic studies, however. Combining data from different groups and laboratories carries considerable challenges. Combining raw data (i.e., mega-analytic approaches) in epigenetics is problematic because technical variation in the data stemming from different laboratories and procedures (i.e., batch effects) has substantial impact on overall epigenomic profiles and can be insufficiently controlled for by post-hoc statistical or computational approaches [58,59]. Nonetheless, approaches in which each site generates a sufficiently large sample under nearly identical conditions that can later be meta-analyzed are feasible [33]. Alternatively, consistent sampling at different study sites including careful preanalytic sample collection and processing followed by analysis in a central laboratory could prevent many of the aforementioned technical issues. However, this approach is only feasible if all study sites meticulously follow the same protocol regarding tissue sampling, sample handling, and phenotyping of participants to control for possible confounders across study sites. This kind of pooling approach tends to be complicated by challenges associated with sample storage, transportation, and loss.

Statistical methods. As with any genome-wide investigation, the large number of tests performed requires special considerations for statistical analysis. Most importantly, it is essential to correct for the number of tests performed. The latest generation of DNA methylation arrays can simultaneously quantify epigenetic profiles at up to 850.000 CpG sites. An EWAS then tests for associations between a phenotype of interest and DNA methylation at each of these sites. Each of the 850.000 tests has a small probability of reporting a false positive association (usually 5%). In order to keep the probability of making any false positive discovery below this probability threshold, the individual P value thresholds for each test need to be adjusted, resulting in a genome- or array-wide significance threshold. This correction for multiple testing can be achieved by common methods such as Bonferroni correction (dividing the P value threshold by the number of tests conducted) or a false discovery rate correction [60,61].

Tissue specificity. Given the prominent role epigenetic mechanisms play in cellular differentiation, genome-wide epigenetic profiles tend to differ substantially between different tissues and cell-types. These considerable differences match the differences in function each cell and tissue have to fulfil. For example, the functions of adipose tissue, such as fat storage, diverge widely from functions such as synaptic transmission performed by neurons in the brain. Different cellular functions require particular sets of proteins acting in concert (i.e., pathways) and epigenetic mechanisms control which genes are active in which cell-type and tissue-context. Interestingly, even within a single tissue like the brain, epigenetic profiles can distinguish functionally different regions (Davies et al., 2012) and cell-types (Lister et al., 2013, Ziller et al., 2015). An important question of interest in epigenetic epidemiology is whether hypothesized disease-associated epigenetic dysregulation is tissue-specific. For example, are epigenetic correlates of psychiatric diseases restricted to the brain? To answer this, it would be ideal to

sample different tissue sites and to examine differential epigenetic profiles per tissue. In some disorders, including AN, for example, it is less straightforward to pinpoint the affected tissue of interest. AN is characterized by both psychiatric and metabolic features [6]. Therefore, one would ideally investigate epigenetic profiles in both brain tissue and metabolic tissues (e.g., adipose tissue, pancreas, liver, stomach, and the intestine). The investigation of brain in particular, however, poses considerable challenges and is typically only possible in postmortem samples, which are accompanied by further difficulties for epigenetic studies (e.g., time of death, cause of death, etc.). Nonetheless, carefully designed, ethical discussions of organ donations with patients and families are worthy of consideration.

Surrogate tissues. Although investigation of epigenetic profiles in the disease-affected tissue is the gold standard in epigenetic studies, it is also valuable to examine epigenetics in surrogate peripheral tissues, including whole blood, epithelial cells, and saliva. First, while epigenetic profiles are highly tissue-specific and profiles observed in peripheral tissues are not generally representative of epigenetic variation in brain, specific genomic regions manifest high levels of epigenetic covariation [62–64]. For example, an existing online platform allows for the profiling of DNA methylation covariation between whole blood and multiple brain regions (<http://epigenetics.essex.ac.uk/bloodbrain/>). Second, while results emerging from epigenetic studies from peripheral samples might not necessarily reflect the epigenetic changes in disorder-relevant tissues, they can still be used as potential biomarkers, and are collected more readily and less invasively than the affected tissue itself. Importantly, when analyzing whole blood epigenetic profiles, the blood cell-type composition also needs to be assessed. Blood is a heterogeneous organ comprised of distinct cell types, fulfilling specific tasks. For instance, in blood, specific cells fulfill functions such as oxygen transport, immune function, and nutrient

distribution. Because blood composition in patients suffering from eating disorders differs from controls [7], it is imperative to control for these differences in cell-type composition. If unaccounted for, epigenetic differences identified in an association scan could be related purely to differences in cellular composition rather than epigenetic dysregulation directly linked to disease etiology and progression. Where blood cell counts are not available, validated estimators of subcell proportions based on large reference panels can be used; i.e. cell-type proportions can accurately be estimated using microarray-based DNA methylation data [65].

Genome-wide integrated epigenetic studies. Many of the studies reviewed here use targeted sequencing approaches, which only allow the investigation of DNA methylation in limited genomic regions and ignore information from the rest of the genome. This may increase the chances for false positive reports via a publication bias of positive findings. Genome-wide technologies are less prone to this phenomenon and allow for the verification of previously reported differentially methylated sites. While only whole genome bisulfite sequencing allows full coverage of the entire genome, array-based approaches like Illumina's EPIC array, allow widespread coverage of CpG sites in most genomic regions and can be a more cost-effective solution.

All epigenetic studies of eating disorders published to date focused on DNA methylation only. A host of other sources of regulatory variation including other DNA modifications, histone modifications, and non-coding RNAs should be investigated, too. Furthermore, to better interpret the role of epigenetic modifications in disease, it is important to understand their interactions with the genetic sequence itself. Integrated analyses incorporating genotypic, epigenetic, transcriptomic, and detailed environmental data are beginning to emerge, elucidating the role of disease-associated epigenetic dysregulation in specific genetic and environmental contexts.

Increasingly detailed maps of genetic and (multi-)epigenetic profiles in health and disease will be essential to improve our understanding of the molecular biological pathways implicated in complex disease.

4.2 Eating disorder specific considerations

In addition to these general recommendations for improving epigenetic research in disease epidemiology, there are also a number of important eating disorder-specific complexities to be considered.

Eating disorder specific confounders. Because epigenetic modifications are dynamic and can be altered by environmental influences, epigenetic association studies are subject to a wide range of confounders. Confounding in EWAS is comparable to classic observational epidemiological studies and ideally these confounders are addressed in the study design in such a way that they can be controlled for in the statistical analyses. For example, age, sex, diet, medication, micronutrients, dietary supplements, smoking, and alcohol consumption can interact with an individual's epigenetic profile, obfuscating EWAS analyses (Figure 2).

Diet. Diet represents a potentially very important confounder in epigenetic studies of eating disorders. A large body of evidence confirms that diet composition can have an effect on an individual's epigenetic profile [66–70]. Eating and compensatory behaviors can include binge eating and purging behaviors, abuse of diet medication, laxatives, and diuretics altering fluid balance. It is important to record their typical use, as well as the frequencies and recency of use and, ideally, obtaining blood levels of diet medications when possible.

Medication and supplements. Although there are few approved pharmaceutical treatments for eating disorders, medication is often prescribed off label. Antidepressants and atypical

antipsychotics are used to control accompanying symptoms observed in patients suffering from eating disorders [71]. Dosage and intake should be included in the analysis, ideally, blood levels should be measured, and statistical analyses corrected for. This strategy should also be followed for supplements, such as vitamins and micronutrients to capture as many factors influencing the epigenetic profile of a patients as possible.

Hormone levels. A further possible confounder in epigenetic studies investigating eating disorders is introduced by hormone levels: Patients with eating disorders often show hormonal alterations, such as high cortisol and low sex hormones [72,73]. These types of hormones are direct ligands to so-called promoters, enhancers, and silencers and, therefore, influence gene expression and protein levels directly. If measured in the same samples, one could account for hormone levels in the statistical analyses. However, if substantial between group differences exist between cases and controls, disease and weight-associated epigenetic variation will remain convoluted. One option in addressing this issue is using a matched weight control group, potentially in addition to normal weight controls, in order to tease apart epigenetic correlates of eating disorder versus altered weight phenotypes. However, this approach may be limited because constitutionally thin individuals rarely have a BMI as low as patients suffering from AN.

Life style factors. Another important consideration, when interpreting epigenetic associations of any complex disease are lifestyle factors, such as smoking and alcohol consumption. As environmental toxins, these substances can have a profound impact on the epigenome. If not accounted for appropriately, these factors can confound EWAS results: for example, prevalence differences in smoking between cases and controls have been shown to confound the association between DNA methylation and schizophrenia [21].

Causal attribution. As with every observational study design, the causal attribution of

epigenetic associations in eating disorders is extremely difficult. Assuming a detected epigenetic association with an eating disorder is real, then it could potentially have causally contributed to the disease; however, the epigenetic dysregulation could also arise as a consequence of the disease, its symptoms or even treatments, such as medication [74]; or, in a third scenario, there could be a third factor driving both the disease and the epigenetic alteration, which have no direct link between one another. One important approach to getting a better understanding of causality is to consider temporal factors [32,33]. A variety of chronologically variable factors should be taken into account, such as age of disorder onset, duration of illness, onset of menstrual disturbances, and duration of amenorrhea (in women). Longitudinal sampling and within-subject comparisons can help differentiate between sequelae of starvation or overeating and epigenetic factors that contribute to the liability to develop an eating disorder. In addition to this, methods using genetic variants as instrumental variables can improve causal inference. In epigenetic epidemiology, Mendelian Randomization is of particular importance, exploiting genetic influences on DNA methylation (mQTLs) to understand whether phenotypic associations of DNA methylation are indeed causal [75].

5. CONCLUSION

Epigenetic research in eating disorders is still in its infancy, but initial results from pilot studies encourage further and larger-scale investigation. Much like progress in genomics, international collaborations are required to amass adequately powered sample sizes to draw credible conclusions from epigenetic investigations. Even more importantly, careful study design is of vital importance in epigenetics and can aid in avoiding potential pitfalls. Robust, replicable results from carefully designed studies have the potential to uncover the molecular biological

processes involved in disease onset and progression, they may help characterize gene regulatory effects of non-coding genetic variation, and, hopefully, give indications into disease-relevant biological pathways which could be addressed by therapeutic interventions. Clearly a considerable amount of functional work is required in follow-up of epigenetic association studies to better understand the gene regulatory, cellular, and organismal outcomes of epigenetic variation and derive potential translational implications and therapeutic avenues. Even non-functional disease-associated epigenetic variation from peripheral tissue sources could, however, have useful implications as biomarkers for risk and prognosis assessment and for use in early diagnosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Figure 1. Epigenetic mechanisms active in the human cell. Histone proteins compact chromosomal DNA in the nucleus of the cell and regulate gene expression. Designed by Vinícius Gaio, London, UK.

Figure 2. Factors and environmental confounders influencing epigenetic profiles.

Figure 3. PRISMA flow diagram of study selection

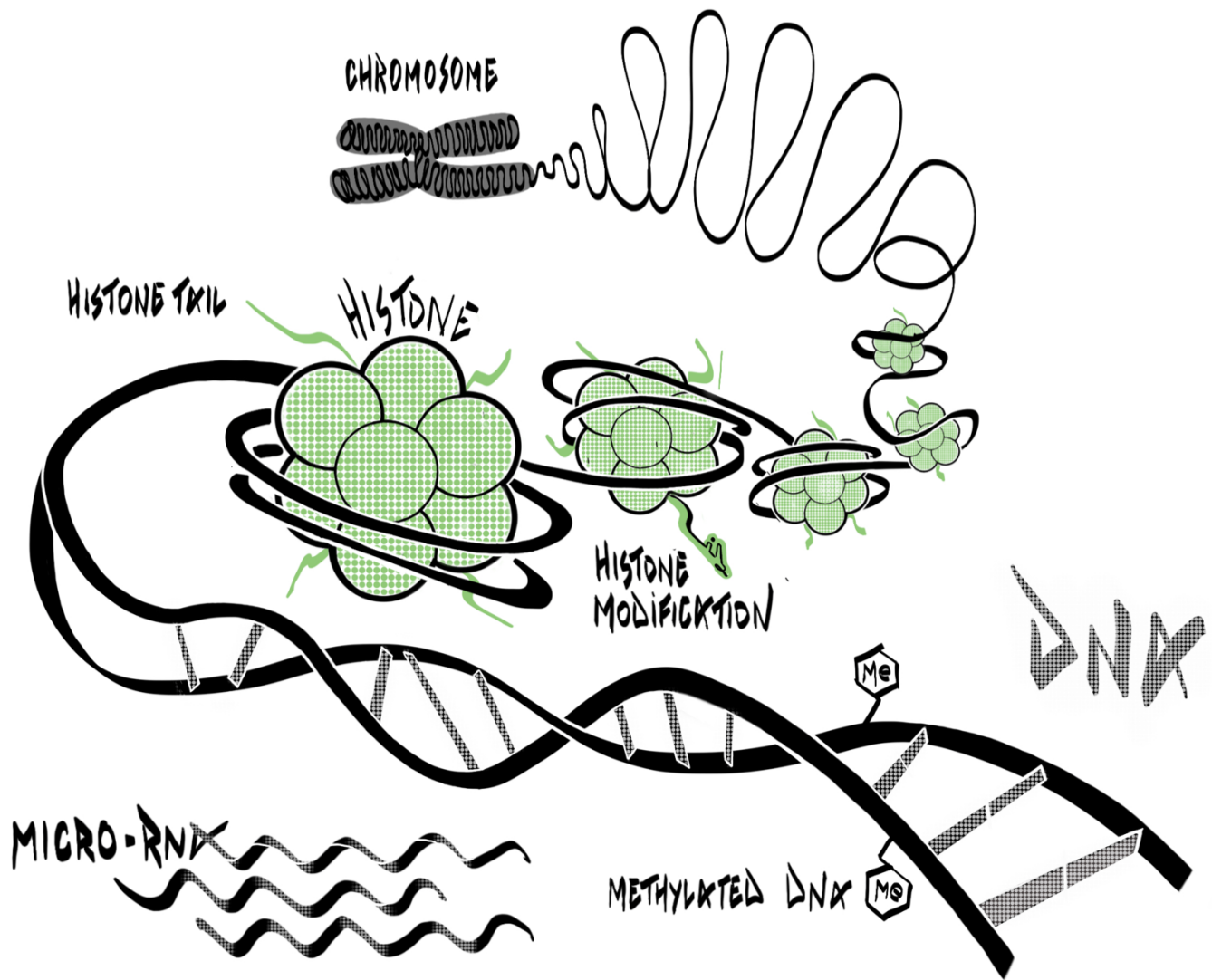
Table 1. Overview of included studies. All studies used DSM-IV or -5 as diagnostic criteria. Tables includes used gene names and also standardized gene names according to www.genenames.org.

Author & Year	Sample	Tissue	Methylation Results			
			Global methylation	AN	BN	BED
Frieling (2007)	AN: 22 BN: 24 CO: 30	Whole blood		↓	↔	
Saffrey (2014)	AN: 10 CO: 10	Buccal cells		↔		
Tremolizzo (2014)	AN: 32 CO: 13	Whole blood		↓		
Booij (2015)	AN: 29 CO: 15	Lymphocytes		↑		
Author & Year	Sample	Tissue	Methylation Results			
			Candidate genes	AN	BN	BED
Frieling (2007)	AN: 22 BN: 24 CO: 30	Whole blood	SNCA HERP	↑ ↔	↔ ↔	
Frieling (2008)	AN: 22 BN: 22 CO: 30	Whole blood	ANP Vasopressin	↔ ↔	↑ ↔	

Frieling (2009)	AN: 20 BN: 23 CO: 26	Whole blood	CNR1/CB1 CB2	↔ n.a.		
Frieling (2010)	AN: 22 BN: 24 CO: 30	Whole blood	SLC6A3/DAT DRD2 DRD4	↑ ↑ ↔	↑ ↔ ↔	
Ehrlich (2010)	AN: 31 AN-Rec: 30 CO: 30	Whole blood	POMC	↔ (AN) ↔ (AN-Rec)		
Ehrlich (2012)	AN: 30 AN-Rec: 21	Whole blood	POMC	↔		
Pjetri (2010)	AN: 45 CO: 45	Whole blood	DRD2 LEP BDNF SLC6A4	↔ ↔ ↔ ↔		
Steiger (2013)	BN: 32 CO: 32	Whole blood	GR		↔	
Groleau (2014)	BN: 206 CO: 102	Whole blood	DRD2		↔	
Kim (2014)	AN: 15 CO: 36	Buccal cells	OXTR	↑		
Thaler (2014)	BN: 64 CO: 32	Lymphocytes	BDNF		↑	

Veldic (2017)	BED/BN: 30 CO: 32	Blood	SLC1A2		↓	↓
Author & Year	Sample	Tissue	Methylation Results			
			EWAS	AN	BN	BED
Booij (2015)	AN: 29 CO: 15	Lymphocytes	NR1H4 PXDNL	2 CpG 3 CpG		
Kesselmeier (2016)	AN: 22 CT: 24 CO: 30	Whole blood	TNXB NR1H3 CSGALNACT1	↑ ↓ SNP		
Ramoz (2017)	AN: 18 AN-Rec: 18	n.a.	n.s.	↔		

AN=anorexia nervosa, AN-Rec=recovered from anorexia nervosa, ANP/NPPA=natriuretic peptide A, BDNF=brain derived neurotrophic factor BED=binge-eating disorder, BN=bulimia nervosa, CNR1/CB1=cannabinoid receptor 1, CNR2/CB2= cannabinoid receptor 2 CSGALNACT1=chondroitin sulfate N-acetylgalactosaminyltransferase 1, DAT/SLC6A3=dopamine transporter, DRD2=dopamine receptor D2, DRD4=dopamine receptor D4, DSM=Diagnostic and Statistical Manual of Mental Disorders, EWAS=epigenome-wide association study GR/NR3C1=glucocorticoid receptor, CO=controls, CT=constitutionally thin, HERP/HERPUD1=homocysteine inducible ER protein with ubiquitin like domain 1, Hcy=homocysteine, LEP=leptin, NR1H3: nuclear receptor subfamily 1 group H member 3, NR1H4=nuclear receptor subfamily 1 group H member 4, NR3C1/GR=nuclear receptor subfamily 3 group C member 1, OXTR=oxytocin receptor PBMC=peripheral blood mononuclear cell, POMC=proopiomelanocortin, PXDNL=peroxidasin like, SLC1A2=solute carrier family 1 member 2 SLC6A3/DAT=solute carrier family 6 member 3, SLC6A4=solute carrier family 6 member 4, SNCA=synuclein alpha, TNXB=tenascin XB





Psychological
state



Diet



Diurnal & seasonal
correlations



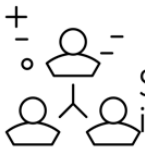
Medication



Chemicals



Development



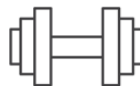
Social
interactions



Microbiome



Diseases



Exercise



Drugs



Alternative
medicine

